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To: R. Carchman

Date: January 7, 1992

From: R. Kinser and R. McCuen

Subject: **Objectives, Strategies and Operational Plans for the Tobacco Biochemistry Major Program**

Plans for the new Tobacco Biochemistry program are attached. All of the individuals in the projects that make up the Program, one Visiting Scientist, and one of the Applied Research Directorate's Staff Scientists, have contributed to the preparation of this document.

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OBJECTIVE #1: To produce a consumer-acceptable cigarette with lowered biological activity.

STRATEGIES:

1. Determine the interactive effects of modifications of conventional cigarette construction on the S/M activity of CSC.

2. Develop the means to reduce undesirable precursors or to enhance desirable precursors.

3. Design a model cigarette whose CSC shows a 90% reduction in S/M activity when compared to the CSC obtained from 2R1 cigarettes.

4. Propose feasibility studies of new developments based on outside literature.

5. Investigate possible constituents of biological activity.

TACTICS AND TIMETABLE OR 1992:

DEVELOP A BASE MODEL FILLER WHICH WOULD HAVE LOW TSNA AND NO BOUND NICOTINE FROM WATER WASHED ORIENTAL FILLER FOR USE IN FURTHER MODEL DEVELOPMENT STUDIES.

Determine feasibility of preparing 500lbs of filler from water washed MT blend

Determine proper inorganics (to assist proper burn characteristics) and obtain analytical information on the cased filler. Deliver half to INBIFO **(Complete by 2nd Q)**

CIGARETTE CONSTRUCTION STUDY

Determine the interactive effect of cigarette construction parameters (blend component, paper porosity and paper additives, ventilation and filter efficiency) on S/M activity of CSC. **(Initiate 1st Q and complete 2nd Q)**

Identify further cigarette construction parameters for a second phase study, such as: various filters and cigarette circumferences **(Initiate 3rd Q)**

Determine feasibility of investigating the effect of peak coal temperature on the S/M activity of CSC **(Initiate 3rd Q)**

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ADDITIVE STUDIES

In coordination with the personnel at INBIFO, continue to study the effect on S/M activity of CSC obtained from cigarettes with the addition of selected amino acids (lysine, threonine, histidine, proline, serine and methionine) to bright or burley solubles (**Initiate 1st Q**)

Identify other compounds as precursors of bioactivity in the S/M assay (**Initiate 3rd Q**)

Determine the feasibility of using strong ion exchange to determine the precursors of S/M activity (**Initiate 3rd Q**)

CONSTITUENTS OF BIOLOGICAL ACTIVITY

Monitor outside scientific literature relevant to biological activity (**Ongoing**)

Obtain probes and implement protocols for analyzing heme oxygenase (HO) mRNA levels and performing nuclear run-on transcription assays in 3T3 cells (**Initiate 1 Q**)

Analyze HO mRNA levels and transcription rates in 3T3 cells which have been subjected to cyclohexamide and TPA treatments (**Initiate 2nd Q**)

Investigate using 2-D gel electrophoresis, 3 or more known growth-regulating proteins before and after treatment with promoters/CSC (**Initiate 1st Q**)

Investigate whether CSC affects expression/levels/sequence of potential key regulation proteins (**Initiate 3rd Q**)

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OPERATIONAL PLANS FOR 1993:

ADDITIVE STUDIES

Determine the feasibility of the use of "desmutagens" in reducing the S/M activity of CSC from cigarettes spiked with precursors. Review external and internal literature to identify potentially useful desmutagens **(Initiate 3rd Q)**

SELECTIVE REMOVAL OF UNDESIRABLE PRECURSORS

Develop methods for the removal of undesirable or precursors of undesirable compounds from water extracts of tobacco by using microorganisms or enzymes **(Initiate 3rd Q)**

BEST MODEL

Use a combination of developed methods to produce a consumer-acceptable model cigarette whose CSC is reduced in S/M activity **(Initiate 3rd Q)**

Establish a protocol for submitting best model cigarette for smoking evaluation **(Initiate 4th Q)**

OBJECTIVE #2: To alter tobacco plants in order to develop consumer acceptable cigarettes or other usable products.

STRATEGIES:

1. Modify tobacco plants to produce the lowest possible levels of alkaloids.

2. Develop tobacco plants with increased resistance to pests.

3. Improve the agronomic, processing or chemical compositional characteristics of tobacco.

4. Develop tobacco plants whose lamina demonstrate increased resistance to pests and microbial degradation during storage.

5. Investigate the usefulness of using tobacco plants as bioreactors.

6. Modify the tobacco plant to maintain MS tobacco alkaloids but reduce other smoke components.

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TACTICS AND TIMETABLE FOR 1992:

A. Modify alkaloid metabolism

1. PMT

   a. Determine additional sequence (**Initiate 1st Q**)

   b. Isolate/identify gene(s) for PMT (**Complete 4th Q**)

      (1) Determine feasibility of using PCR (**2nd Q**)
      (2) Southern blot analysis of genomic DNA with probes for PMT (**2nd Q**)
      (3) Determine feasibility of using nonradioactive detection methods for isolation/identification of PMT (**Initiate 1st Q**)

   c. Screen expression library using Ab to N-terminal sequence of PMT (**Initiate 2nd Q**)

   d. Make anti-sense constructs with cDNA sequence obtained from antibody screen (**Initiate 3rd Q**)

   e. Obtain transgenic tobacco plants after transformation with anti-sense DNA (**Ongoing**)

   f. Analyze transgenic tobacco plants for evidence of anti-sense cDNA incorporation and expression (**Initiate 1st Q**)

2. Use differential hybridization to look for additional root specific genes (**Initiate 1st Q but only if visiting scientist is recruited**)

3. Begin the isolation of N-methylputrescine oxidase (**Initiate 1st Q**)

   a. Develop MPO assay (**1st Q**)

   b. Determine characteristics of MPO (**Initiate Feb. 1**)

      (1) Standard biochemical measurements
      (2) Inhibition studies

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c. Isolate MPO

(1) Assay Phenyl-Sepharose fraction for MPO activity (**2nd Q**)
(2) Examine anion exchange column(s) (**Initiate 2nd Q**)
(3) Examine metal chelate chromatography (**Initiate 1st Q**)
(4) Examine HPLC chromatographic column methodologies (**2nd Q**)
(5) Examine via 2-D analysis (**3rd Q**)

d. Examine enzyme stability

(1) Activity staining methods (**Initiate 1st Q**)
(2) In presence of various compounds (**Initiate Feb. 1**)

4. Begin isolation of Nicotine Synthetase (**Initiate 1st Q**)

5. Begin work on the use of nicotine degrading enzymes and their respective coding genetic responses (**Initiate 1 Q**)

6. Identify outside facility for implementation of transgenic activities (**4th Q**)

7. Investigate feasibility of altering tobacco plant to yield a cigarette with normal MS nicotine delivery but minimal delivery of other organic components (**Initiate 4th Q**)

TACTICS AND TIMETABLE FOR 1993:

A. Pest resistance

1. Present a comprehensive review of the Bacillus thuringiensis literature to management (**1st Q**)

2. In collaboration with FTR personnel develop a screen for prokaryotic organisms which have activity against CBs (**1st and 2nd Q**)

3. In collaboration with FTR personnel screen prokaryotic organisms for activity against CBs (**2nd Q and continuing**)

B. Improve agronomic, processing or chemical composition characteristics.

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1. Determine those agronomic, processing or chemical compositional characteristics whose changes will result in an improved product precursor/ product. Determine feasibility of altering those characteristics. If feasible, prepare a memo outlining a course of action based on that determination (**Complete by the end of 3rd Q**)

C. Improve storage stability

1. Determine those physical/biochemical characteristics which would improve the long-term storage of tobacco. If feasible to alter/modify those characteristics, prepare a a memo outlining that course of action based on that determination (**Complete by the end of 3rd Q**)

D. Investigate tobacco cells as bioreactors

1. Determine the potential PM needs of any expensive/difficult-to-obtain/ seasonal compounds/biochemicals/proteins/ plant cell materials compound. Determine the feasibility of producing those compounds using genetically/biochemically modified tobacco or tobacco cell tissue culture. If feasible, prepare a memo documenting a course of action based on that determination (**Complete by the end of 4th Q**)

OBJECTIVE #3: To use biological systems to produce usable products.

STRATEGY

1. Use modified plant/plant tissue culture/bacterial systems to produce flavors/chemicals/materials which can be of use to PM.

TACTICS AND TIMEBABLE FOR 1992:

A. Produce compounds of interest to PM.

1. Determine the potential PM needs of any expensive/difficult-to-obtain/seasonal compounds/biochemicals/proteins/plant cell materials. Determine the feasibility of producing those compounds using genetically/biochemically modified tobacco or tobacco cell tissue culture. If feasible, prepare a memo documenting a course of action based on that determination (**Complete by the end of 4th Q**)

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OBJECTIVE #4: To produce consumer-acceptable cigarettes with reduced levels of undesirable compounds.

STRATEGIES:

1. Devise methods to reduce MS NNK formed from bound precursors during smoking.

2. Reduce MS TSNA by modification of cigarette construction parameters.

3. Reduce MS TSNA by selective physical removal of TSNA and TSNA precursors.

4. Reduce MS TSNA by adding inhibitors of nitrosation or agents to decrease transfer of preformed TSNA.

5. Reduce MS TSNA by use of tobaccos naturally low in preformed TSNA, minor alkaloids, and/or nitrosating agents.

6. Investigate PM processing methods to determine potential for modifications that would reduce MS TSNA.

7. Determine compounds of interest as candidates for reduction in cigarette smoke.

TACTICS AND TIMETABLE FOR 1992:

MS NNK REDUCTION STRATEGY:

Verify relationship between bound Nicotine and bound NNK:

Determine time course of bound nicotine and bound NNK formation during air-curing:

Measure levels of NIC-X, NIC-Y and bound NNK in greenhouse and field grown Bu 21 **(Initiate 1st Q)**

Evaluate results **(Complete 2nd Q)**

Measure levels of NIC-X and NIC-Y in various tobacco fillers **(Ongoing)**

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Isolation and structures of different forms of bound nicotine and bound NNK:

Development of isolation methods for NIC-X, NIC-Y & bound NNK:

Chromatography of extracts from ART Bu **(Initiate 1st Q)**

Enrichment by acid digestion of marc from hot water wash of ART Bu **(Initiate 2nd Q)**

Enzyme digestion of acid marc **(Initiate 2nd Q)**

Remove cellulose from ART Bu (N-Methylmorpholine-N-oxide digestion) **(Initiate 2nd Q)**

Determine chirality of nicotine liberated by alkaline digestion **(Initiate 1st Q)**

Evaluate structures (nature of substrates) of bound materials **(Complete 4th Q)**

Synthesis of bound nicotine and bound NNK:

In vitro coupling of nicotine to known and/or extracted phenolics (synthetically bound nicotine **(Initiate 1st Q)**

Enzyme-catalyzed synthesis of bound nicotine: Reaction of nicotine with phenylpropanoids + peroxide **(Initiate 2nd Q)**

Investigate the oxidation and nitrosation of synthetically bound nicotine, followed by release of NNK *via* pyrolysis or alkaline digestion **(Initiate 3rd Q)**

Determine the structure of synthetically bound nicotine **(Complete 4th Q)**

Study the release of MS NNK from preformed bound NNK during smoking:

Determination of alkali-releasable NNK from water-extracted fillers **(Complete 2nd Q)**

Development of pyrolytic means of liberation of NNK from bound NNK sources such as water-extracted fillers, isolated bound nicotines **(Complete 2nd Q)**

Determination of NNK releasable from various marcs and isolated/synthesized sources of bound NNK **(When available)**

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CIGARETTE CONSTRUCTION PARAMETER STRATEGY:

Systematic (e.g. response-surface-studies) evaluation of cigarette construction/MS TSNA delivery:

Determine MS TSNA/dry TPM and obtain CTSD data for cigarettes used in above study, with plugs pulled/holes taped **(Complete 2nd Q)**

Determine MS TSNA/dry TPM for the construction parameters included in above study and compare data with the same ratio determined with no dilution or filter; use as predictor for MS TSNA of a given cigt. **(Complete 2nd Q)**

REDUCTION OF MS TSNA BY PHYSICAL REMOVAL OF PREFORMED TSNA OR THEIR PRECURSORS, OR BY ADDING INHIBITORS:

1st Generation Low-TSNA Laboratory Model:

Evaluate potential for product development of the 1st Generation Low-TSNA Model **(1st Q)**

If there is potential, reconstruct the Low-TSNA Blend developed using ethanol extraction **(2nd Q)**

Transfer technology for Low-TSNA Blend to Flavor Development for final formulation and panel testing **(2nd Q)**

Further process development for removing bound NNK and other MS TSNA precursors:

Determine the feasibility of using supercritical CO_2 modified with a polar additive for removal of TSNA and secondary amine alkaloids **(Initiate 1st Q)**

Determine the feasibility of using a supercritical fluid other than CO_2 for removal of TSNA and secondary amine alkaloids **(Initiate 2nd Q)**

Develop a low-TSNA model based on a water-washed filler to which the concentrated washings are applied after removal of alkaloids & TSNA *via* cation exchange **(Initiate 2nd Q)**

Devise means to reduce MS NNK derived from bound NNK based on the best information at hand **(Complete 4th Q)**

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Use all information available to establish the lowest possible MS TSNA delivery in a full flavored blended cigarette.

2nd Generation Low-TSNA Laboratory Model:

Formulate a new Low-TSNA Model based on technology determined to date **(Complete 4th Q)**

USING TOBACCOS NATURALLY LOW IN PRODUCTION OF MS TSNA STRATEGY:

Investigate the possibility of utilizing tobaccos which are naturally low in production of MS TSNA:

Obtain samples of burley grades from 1988, 1989, and 1990 crop years and determine preformed and MS TSNA levels as well as alkaloids, etc. **(Complete 1st Q)**

Compare DBC burley versions for the three years studied with the above data and determine whether there are individual grades that make significant contributions to MS TSNA of the blend **(Complete 2nd Q)**

Repeat the above for bright **(Initiate 2nd Q)**

Continue interaction with Leaf to monitor available grades **(Ongoing)**

PROCESSING METHODS STRATEGY:

Study existing PM processing techniques to determine practical approaches for reducing MS TSNA:

Assemble all data available on the effects of PM processes on MS TSNA delivery **(Initiate 1st Q)**

Obtain data on other processes believed to have potential impact on MS TSNA **(Ongoing)**

Determine possible steps to reduced TSNA based on an examination of all the above data **(Complete 3rd Q)**

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Determine effect of adding antioxidants to SEL on MS TSNA delivery of Pilot Plant RL (**Initiate 2nd Q**)

Determine the effect of removing nitrate from BuCEL *via* electrodialysis on MS TSNA from BuCEL/BrBW (**1st Q**)

ANALYTICAL METHODS DEVELOPMENT **(Support for above strategies):**

Replace one HP 5790 GC with a HP 5890 GC, and establish protocol for computer-operated GC/TEA analysis (**Complete 1st Q**)

Improve GC parameters used for GC/TEA (**Ongoing**)

Develop an SCFE method for filler TSNA (**Complete 3rd Q**)

TACTICS AND TIMETABLE FOR 1993:

Determine whether there are significant sources of MS NNK other than bound NNK and transfer of preformed NNK; e.g., pseudooxynicotine (PsON) or N-methylmyosmine (NMM)

Determine if there is interest in further development of the 2nd Generation Low-TSNA Model.

> *if yes:* transfer technology for new Low-TSNA model to Development for further formulation and panel testing (**3rd Q**)

Propose methods for reduction of other undesirable compounds.

Continue to monitor available grades of specific blend components for TSNA levels.

Continue to evaluate PM processing methods and propose studies to determine methods for reducing MS TSNA.

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